[25-Oxindolylalanine]glucagon and [27-Methionine sulfoxide]glucagon: Preparation, Purification, and Characterization†

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ABSTRACT: Glucagon analogues selectively oxidized at the hydrophobic 25-tryptophan and 27-methionine residues were prepared in >99% purity, generating more polar derivatives containing 25-oxindolylalanine and 27-methionine sulfoxide. Reaction of native glucagon with Me_2SO in 12 N HCl oxidizes the tryptophan and methionine residues to give [25-oxindolylalanine,27-methionine sulfoxide]glucagon. After reduction of the methionine sulfoxide with mercaptoacetic acid, purification by cation-exchange chromatography at pH 5.1 gave a homogeneous [25-oxindolylalanine]glucagon product having a p*I* of 6.5, identical with that of native glucagon. [27-Methionine sulfoxide]glucagon was prepared by reacting native glucagon with Chloramine T in Tris-HCl, pH 10.0. Methylation of the reaction mixture converts unreacted glucagon to the positively charged sulfonium derivative, [27-*S*-methylmethionine]glucagon, which was separated by cation-exchange chromatography. Alternatively, cyanogen bromide cleavage of unreacted methionine followed by ring opening of the homoserine lactone permits quantitative assessment of methionine sulfoxide purity. [27-Methionine sulfoxide]-glucagon was shown to be homogeneous upon isoelectric focusing with a p*I* of 6.5, identical with that of native glucagon. Binding affinity, measured by displacement of [^{125}I]iodoglucagon, and activation of adenylate cyclase of rat liver plasma membranes were identical with those of native glucagon for both oxidized derivatives. The derivatives failed to show a concentration-dependent increase in helicity at alkaline pH typical of the native hormone as shown by circular dichroism. These findings indicate that these modifications do not interfere with binding and activation and that forces other than the hydrophobic ones provided by the side chains of 25-tryptophan and 27-methionine are important for these biological functions. The results suggest that the oxidizing conditions used to label native glucagon yield derivatives with binding and activation data representative of that expected for the native hormone.

Glucagon, a 29 amino acid peptide hormone, is highly conserved throughout mammalian evolution, suggesting that the entire structure of the molecule is required for its full biological expression (Sundby, 1976). Interaction of glucagon with specific receptors on plasma membranes of target cells results in the stimulation of adenylate cyclase, ultimately leading to a variety of biological responses (Pohl et al., 1969; Rodbell et al., 1971). This interaction with specific receptors has been thought to be facilitated by hydrophobic clusters at the amino and carboxyl terminal of the molecule (Sasaki et al., 1975). Crystallographic studies have shown that these hydrophobic clusters interact in the self-aggregation that occurs when trimers are formed and presumably when aggregation occurs at elevated solution concentration.

Studies with glucagon fragments reveal a functional asymmetry to the molecule in that the amino terminal contributes somewhat to the binding process but is more important in the expression of hormone action, while the carboxyl terminal is most important in the process of recognition at the receptor (Rodbell et al., 1971; Lin et al., 1975; Jones & Gurd, 1981; England et al., 1982). In solution this carboxyl-terminal region is believed responsible for the residual helical structure characteristic of the molecule (Sasaki et al., 1975), although a nonhelical nucleation site has also been postulated to exist in this region (Bösch et al., 1978).

It has been demonstrated that the carboxyl region contributes significantly to the ultimate receptor binding process as indicated by specific modifications involving the 27-methionine, 28-asparagine, and 29-threonine residues (Epand, 1972; Lin et al., 1975; Jones & Gurd, 1981; England et al., 1982) and also by the fact that glucagon$_{1-21}$, lacking the entire carboxyl-terminal region, requires a 1000-fold higher concentration to yield 50% adenylate cyclase activity than does native glucagon (Wright et al., 1978). In addition, particular interest in the carboxyl-terminal hydrophobic patch derives from the damage incurred in this region during the iodination of the hormone by Chloramine T for radioligand-displacement studies and the report that oxidation of methionine diminishes the glucagon–antiglucagon antibody interaction of the carboxyl-terminal portion of glucagon (Shima et al., 1975). Oxidation has also been reported to perturb glycogenolysis in and binding to cultured cells (Nooijen & Kempen, 1979; Sonne et al., 1982).

This report details the preparation, isolation, and chemical characterization of two oxidation products of the hormone, [25-oxindolylalanine]glucagon and [27-methionine sulfoxide]glucagon. Furthermore, the ability of these two derivatives to form helical aggregates at alkaline pH has been examined by circular dichroism. The derivatives are compared with native glucagon and [27-*S*-methylmethionine]glucagon with respect to their binding to partially purified rat liver plasma membranes as well as their ability to activate adenylate cyclase of these membranes.

Experimental Procedures

Materials

Crystalline porcine glucagon was provided through the courtesy of Eli Lilly and Co. (Lot No. 258-D30-138-2). Methanesulfonic acid (4 N) containing 3-(2-aminoethyl)indole

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was purchased from Pierce Chemical Co., and "ultrapure"-grade Tris–base was obtained from Schwarz/Mann. Copper-free methyl iodide was obtained from Mallinckrodt and Chloramine T from Eastman Kodak. Dithiothreitol and leucine aminopeptidase (microsomal) came from Sigma Chemical Co.

The G-10 and G-25F gel filtration resins were obtained from Pharmacia, and the CM-cellulose (CM-52) ion-exchange resin was from Whatman. The materials required for isoelectric focusing came from Bio-Rad Laboratories. All other chemicals were reagent grade and were used without further purification. Distilled, deionized water was used throughout. In all preparations containing urea, the urea solution was deionized on a mixed-bed ion-exchanger Rexyn I-300 column (2.5 × 50 cm) immediately before use.

Methods

Amino Acid Analysis. Enzymatic digests were performed with leucine aminopeptidase (microsomal) at 37 °C for 24 h in 0.05 M phosphate buffer, pH 7.8, containing 0.5 mg/mL chloramphenicol. A blank run was also analyzed. Routine acid hydrolysates were prepared in 6 N HCl at 110 °C for 24 h and were run on a Beckman 120 amino acid analyzer equipped with Durrum DC-6A resin (Jones & Gurd, 1981). Oxindolylalanine, tryptophan, and *S*-methylmethionine were analyzed on a column (0.9 × 19 cm) of Beckman PA-35 resin following hydrolysis at 110 °C for 24 h in 4 N methanesulfonic acid containing 3-(2-aminoethyl)indole (Simpson et al., 1976; Savige & Fontana, 1977). Homoserine was differentiated from homoserine lactone by its elution position on the DC-6A column as previously described (Jones & Gurd, 1981). The analyzer systems were interfaced with a Texas Instruments 980A minicomputer that performed the identification and integration of the amino acid chromatograms (Jones et al., 1978).

Isoelectric Focusing. Isoelectric focusing was carried out as described in the instructions provided by Bio-Rad Laboratories by using 7.5% polyacrylamide tube gels (5 × 110 mm) containing 6 M urea and 2% (w/v) pH 3–10 carrier ampholytes. A pH profile was determined by placing 0.5-cm sections of a blank gel in water for 1 h and then measuring the pH. Protein bands were located by staining with Coomassie Brilliant Blue G250 (Blakesley & Boezi, 1977).

Circular Dichroism. CD[1] measurements were performed in 0.2 M Na_2HPO_4 (pH 10.2) at varying protein concentrations as previously described (Rothgeb et al., 1978). Samples were repetitively scanned at 3 nm/min with a time constant of 4 s. The spectra were analyzed by the method of Greenfield & Fasman (1969) using the reference spectra of Chen et al. (1974) taken at 1-nm intervals over the range from 240 to 205 nm. Peptides were dissolved at approximately 3 mg/mL and serial dilutions were made from the stock solution.

Biological Assays. Binding and adenylate cyclase assays were performed as described by England et al. (1982), except that the incubation buffer used for the glucagon binding assay was 30 mM Tris-HCl, pH 7.5, 1 mM DTT, and 0.1% BSA. The concentration of the [27-methionine sulfoxide]glucagon was determined spectrophotometrically in the incubation buffer after Millipore filtration of the sample. The 278-nm molar absorptivity was taken to be 8260 M^{-1} cm^{-1} (Gratzer & Beaven, 1969). Due to the decreased absorbance of the oxindolylalanine residue at 278 nm, the concentration of [25-oxindolylalanine]glucagon was determined by amino acid analysis. The peptide concentration was taken to be an average of the amino acids eluting from the DC-6A column based upon the known sequence of porcine glucagon. Aspartic acid, threonine, and serine, whose values are characteristically low due to partial destruction during acid hydrolysis, were omitted from the average.

Purification of Native Glucagon. Glucagon was purified following the procedures described by Flanders et al. (1982).

Preparation of [27-S-Methylmethionine]glucagon. [27-*S*-Methylmethionine]glucagon was prepared and purified following the procedures described by Rothgeb et al. (1977).

Preparation of [25-Oxindolylalanine]glucagon. [25-Oxindolylalanine]glucagon was prepared as outlined by Savige & Fontana (1977). Purified native glucagon (10 mg) was dissolved in a mixture of 1.0 mL of glacial acetic acid and 0.5 mL of 12 N HCl. Dimethyl sulfoxide (Me_2SO, 40 μL, 200-fold excess) was then added and the reaction mixture stirred in the dark at room temperature for 30 min. The reaction was terminated by the addition of 1.0 mL of deionized water and the mixture loaded directly onto a 1.6 × 110 cm Sephadex G-25 column equilibrated with 5% acetic acid. The column was eluted with 5% acetic acid and monitored for both conductivity and absorbance at 278 nm. The peptide fraction was pooled and lyophilized. Selective reduction of the single methionine residue oxidized during the above procedure was accomplished with mercaptoacetic acid. The oxidized reaction mixture (5 mg) was dissolved in 1.0 mL of water containing 0.2 mL (2.90 M) mercaptoacetic acid. The solution was adjusted to a final pH of 2.3 with 1 N NaOH and stirred at room temperature for 48 h (Houghten & Li, 1979). The reaction mixture was desalted on Sephadex G-25 as described above and lyophilized.

The above reaction mixture was then purified on a 1.6 × 15 cm column of Whatman CM-52 cation-exchange resin equilibrated with a first buffer of 10 mM ammonium acetate in 6 M urea, pH 5.1. The peptides were eluted at 30 mL/h with a 24-h linear gradient formed between the first buffer and a second buffer of 50 mM ammonium acetate in 6 M urea, pH 5.1. The effluent was continuously monitored for conductivity and absorbance at 278 nm. The peptide fraction eluting at the position of native glucagon was desalted on Sephadex G-25 and lyophilized.

Preparation of [27-Methionine sulfoxide]glucagon. [27-Methionine sulfoxide]glucagon was prepared following the procedures of Shechter et al. (1975). Purified native glucagon (10 mg) was dissolved in 5.0 mL of a 0.1 M Tris-HCl buffer, pH 10.0. A 30-fold molar excess of Chloramine T (24.2 mg dissolved in 0.5 mL of 0.1 M Tris-HCl buffer, pH 10.0) was added and the reaction mixture stirred in the dark at room temperature for 5 min. The reaction was terminated with a 30-fold molar excess of mercaptoethanol (6 μL) and the mixture loaded directly onto a 1.6 × 100 cm Sephadex G-10 column equilibrated and eluted with 0.05 M ammonium bicarbonate. The eluate was monitored for conductivity and absorbance at 278 nm. The peptide fraction was pooled and lyophilized.

Methylation of the 27-Methionine Sulfoxide Reaction Mixture. The [27-methionine sulfoxide]glucagon reaction mixture was methylated following the procedures outlined by Rothgeb et al. (1977), desalted on a Sephadex G-25 column as described above, and lyophilized for subsequent characterization.

[1] Abbreviations: BNPS-skatole, 2-[(2-nitrophenyl)sulfenyl]-3-bromo-3-methylindolenine; BSA, bovine serum albumin; CD, circular dichroism; CNBr, cyanogen bromide; Me_2SO, dimethyl sulfoxide; DTT, dithiothreitol. Other abbreviations are those recommended by the IUPAC–IUB.

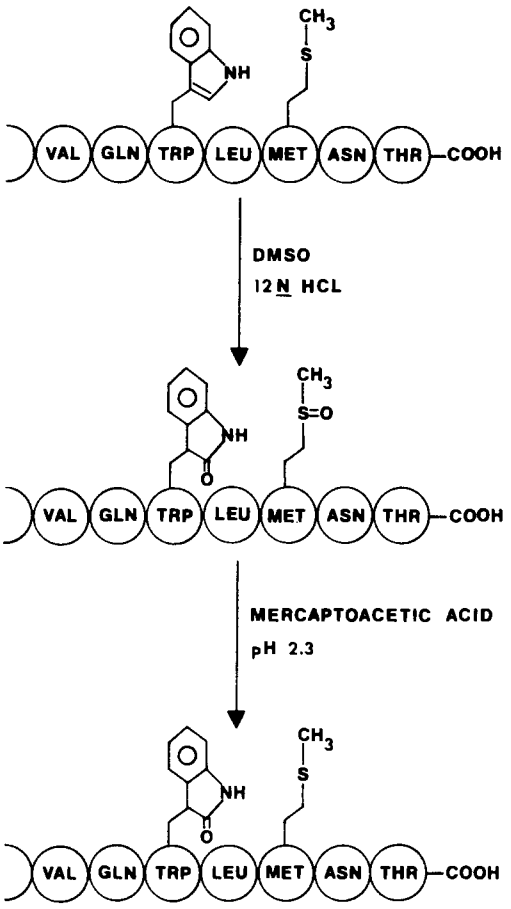

FIGURE 1: Summary of the reaction scheme employed to generate [25-oxindolylalanine]glucagon. Details are given in the text. DMSO is dimethyl sulfoxide.

The methylated, [27-methionine sulfoxide]glucagon reaction mixture was resolved on a 1.6 × 15 cm column of Whatman CM-52 cation-exchange resin equilibrated with a first buffer of 10 mM ammonium acetate in 6 M urea, pH 4.5. The column was eluted at 30 mL/h with a 24-h linear gradient formed between the first buffer and a second buffer of 100 mM ammonium acetate in 6 M urea, pH 5.4. The effluent was monitored continuously for both conductivity and absorbance at 278 nm. The peptide fraction eluting at the position of native glucagon was desalted on Sephadex G-25 and lyophilized.

Cyanogen Bromide Cleavage of [27-Methionine sulfoxide]glucagon. Aliquots of the purified [27-methionine sulfoxide]glucagon were treated with a 200-fold excess of cyanogen bromide in a 70% formic acid solution for 48 h at 4 °C in the dark (Jones & Gurd, 1981). The cyanogen bromide and formic acid were removed by lyophilization. After routine acid hydrolysis in 6 N HCl, any homoserine lactone present was converted to homoserine by adding pyridine–acetate buffer, pH 6.5, and heating at 110 °C for 1 h. The buffer was removed by lyophilization and the amino acid composition analyzed.

Results

Preparation of [25-Oxindolylalanine]glucagon with Me_2SO. The reaction scheme in Figure 1 illustrates the oxidation of native glucagon with Me_2SO in 12 N HCl to form [25-oxindolylalanine,27-methionine sulfoxide]glucagon. Me_2SO in HCl was chosen due to its relatively mild oxidizing potential compared with *N*-bromosuccinimide or BNPS-skatole. This procedure is reported to avoid cleavage of the tryptophanyl peptide bond or overoxidation of the residue to form dioxindolylalanine (Savige & Fontana, 1977). The methionine sulfoxide was reduced with mercaptoacetic acid to generate [25-oxindolylalanine]glucagon (Houghten & Li, 1979) in order to differentiate which amino acid was responsible for any small differences noticed in subsequent characterizations.

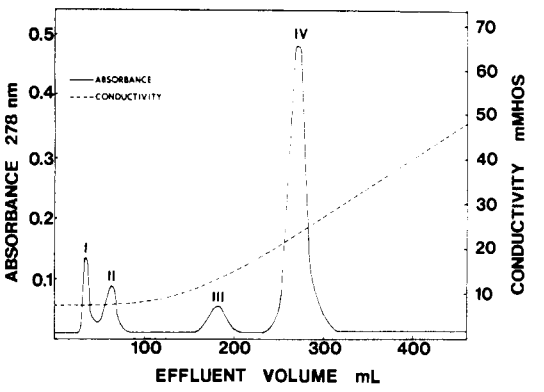

FIGURE 2: Elution profile from CM-cellulose cation-exchange chromatography of the [25-oxindolylalanine]glucagon reaction mixture. Details are given in the text.

Table I: Amino Acid Composition of [25-Oxindolylalanine]-glucagon and [27-Methionine sulfoxide]glucagon

amino acid	[25-oxindolylalanine]glucagon acid hydrolysis[a]	[27-methionine sulfoxide]glucagon enzymatic hydrolysis
Asp	3.72[c] (4)[b]	3.21 (3)
Thr	2.92[c] (3)	3.47 (3)
Ser	4.00[c] (4)	4.06 (4)
Glu	2.93 (3)	<0.01 (0)
Gly	1.10 (1)	1.11 (1)
Ala	1.17 (1)	1.26 (1)
Val	1.00 (1)	1.02 (1)
Met	0.94 (1)	<0.01 (0)
Leu	2.04 (2)	2.13 (2)
Tyr	1.87 (2)	1.95 (2)
Phe	1.92 (2)	1.98 (2)
Lys	0.95[e] (1)	1.08[e] (1)
His	0.96[e] (1)	0.80[e] (1)
Arg	2.08[e] (2)	2.04[e] (2)
Trp	<0.01[e] (0)	1.17[e] (1)
OIA[d]	0.92[e] (1)	<0.01[e] (0)

[a] Methanesulfonic acid hydrolysis. [b] Expected values in parentheses are based on the glucagon sequence. [c] Values corrected for partial destruction during acid hydrolysis. [d] OIA = oxindolylalanine. [e] These values were obtained from analysis on the PA-35 column.

Figure 2 shows the elution profile for the purification of [25-oxindolylalanine]glucagon by cation-exchange chromatography. Purification of the reaction mixture removes the minor products of nonspecific deamidation and other undesired products (peaks I–III, which were not further characterized). Peak IV was shown to be [25-oxindolylalanine]glucagon by amino acid analysis on PA-35 resin following acid hydrolysis (Table I). As can be seen, tryptophan was essentially absent whereas one residue of oxindolylalanine was quantitated directly from its peak that elutes just prior to tryptophan on this resin (Savige & Fontana, 1977). Purity was subsequently determined from the tryptophan content of the sample following overloading of the analyzer. Under these conditions, less than 1 nmol of tryptophan in 150 nmol of peptide hydrolysate remained, indicating a purity greater than 99%. [25-Oxindolylalanine]glucagon was found to be homogeneous upon isoelectric focusing and cofocused with native glucagon at a p*I* of 6.5.

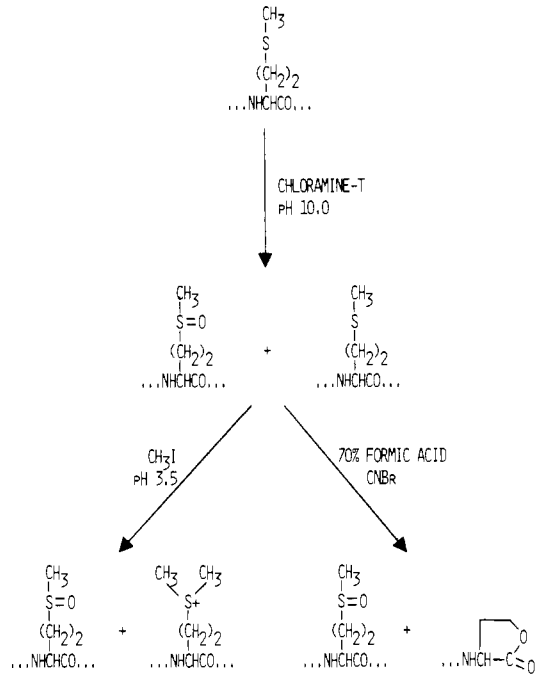

FIGURE 3: Summary of the reaction schemes employed to generate and identify [27-methionine sulfoxide]glucagon. Details are given in the text.

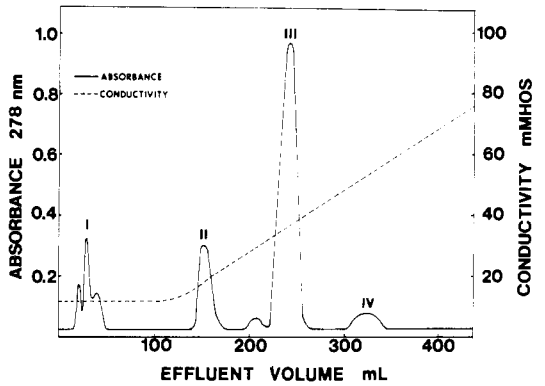

FIGURE 4: Elution profile from CM-cellulose cation-exchange chromatography of the [27-methionine sulfoxide]glucagon reaction mixture. Details are given in the text.

Preparation of [27-Methionine sulfoxide]glucagon with Chloramine T. Figure 3 indicates the oxidation of the sole methionine by Chloramine T. Although methionine residues in ribonuclease may also be selectively oxidized with Me_2SO in 6 N HCl rather than 12 N HCl (Savige & Fontana, 1977), this procedure has proved unsatisfactory for glucagon, yielding incomplete oxidation of the methionine or concomitant oxidation of the tryptophan. Therefore, we have followed the procedures of Shechter et al. (1975) utilizing Chloramine T as the oxidizing agent. As shown in Figure 3, the oxidation of native glucagon by Chloramine T was carried out at pH 10.0. This pH was selected in order to minimize the concomitant oxidation of tryptophan that occurs at pH values below 7.0 (Shechter et al., 1975). In order to remove any unreacted native glucagon present in the reaction mixture, the highly efficient methylation reaction was carried out, forming the positively charged sulfonium derivative, [27-*S*-methylmethionine]glucagon, from any residual unreacted products. The reaction mixture was purified by cation-exchange chromatography, which removes the products of nonspecific deamidation as shown in Figure 4 (peaks I and II, which were not further characterized). Peak IV eluted in the chromatographic position expected for the positively charged derivative, [27-*S*-methylmethionine]glucagon, and was identified as such by amino acid analysis (data not presented). The major fraction (peak III) eluted in the position expected for native glucagon and was shown to be [27-methionine sulfoxide]-glucagon by amino acid analysis (Table I). The absence of methionine indicates that 99% of the amino acid has been modified but does not differentiate whether the product formed is the sulfoxide or the sulfone. In control studies of the oxidation of free methionine, methionine sulfoxide was shown to elute just prior to aspartic acid on the DC-6A resin; however, in peptide hydrolysates, methionine sulfoxide was not sufficiently resolved to allow accurate quantitation of the residue. Subsequently, in order to exclude the possibility of overoxidation to form methionine sulfone, samples of the derivative were hydrolyzed in 6 N HCl, and the methionine content was determined. Since the sulfone is stable during acid hydrolysis while the sulfoxide is reduced back to methionine, the sulfone may be quantitated from the lack of methionine recovered (Means & Feeney, 1971). Following acid hydrolysis of the oxidized hormone under reducing conditions, 0.99 residue of methionine remained, suggesting that less than 1% of methionine sulfone was present in the sample.

Table II: Amino Acid Composition of Native Glucagon and [27-Methionine sulfoxide]glucagon following Treatment with CNBr

amino acid	native glucagon acid hydrolysis	[27-methionine sulfoxide]glucagon acid hydrolysis
Asp	4.12[b] (4)[a]	4.39[b] (4)
Thr	2.89[b] (3)	2.97[b] (3)
Ser	3.40[b] (4)	3.66[b] (4)
Glu	2.76 (3)	2.72 (3)
Gly	1.00 (1)	1.05 (1)
Ala	1.03 (1)	0.98 (1)
Val	0.96 (1)	0.96 (1)
Met	<0.01 (0)	1.06 (1)
Leu	1.82 (2)	1.88 (2)
Tyr	1.69 (2)	1.70 (2)
Phe	1.87 (2)	1.88 (2)
Lys	1.01 (1)	1.07 (1)
His	1.01 (1)	1.08 (1)
Arg	2.19 (2)	2.21 (2)
Trp	ND[c]	ND
Hse[d]	0.99 (1)	<0.01 (0)

[a] Expected values in parentheses are based on the glucagon sequence. [b] Values corrected for partial destruction during acid hydrolysis. [c] ND = not determined. [d] Hse, homoserine.

To define further the oxidation state of the modified methionine, we employed an alternative method for determining the methionine sulfoxide content. This strategy involves cleavage of the derivative with cyanogen bromide at 27-methionine, thereby removing the dipeptide asparaginyl-threonine, and converting the methionine to homoserine or homoserine lactone (Jones & Gurd, 1981). In analogy with the protection of the methionine residue by methylation, protection of the methionine by oxidation prevents cleavage. Therefore, the purified oxidized sample was cleaved with cyanogen bromide, hydrolyzed in 6 N HCl, and then treated with pyridine–acetate. Acid hydrolysis converts the methionine sulfoxide to free methionine, while treatment with pyridine–acetate opens the lactone ring of any homoserine present as a result of the cleavage. Table II shows the amino acid analysis of native glucagon and [27-methionine sulfoxide]glucagon following cyanogen bromide cleavage. As expected, the methionine content of native hormone following this cleavage was essentially zero while the methionine content of the oxidized

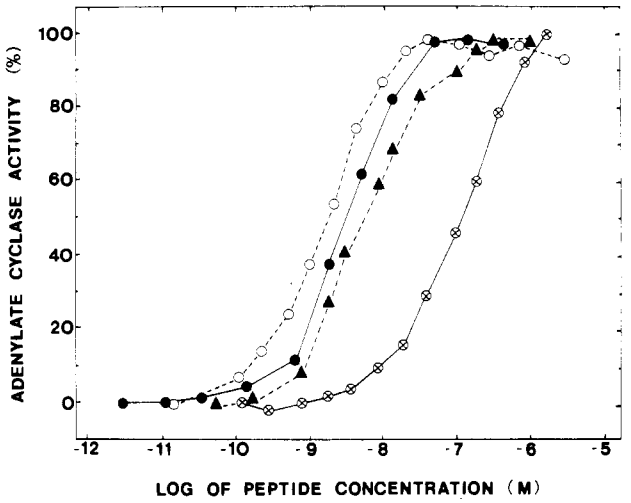

FIGURE 5: Dose–response curves for the activation of rat liver adenylate cyclase. Results are expressed as the percent of activation over the basal level, which averaged about 0.4 nmol of cAMP formed/mg of membrane protein in 10 min. Maximum activity was 3–4 times the basal level activity (England et al., 1982). (●) Native glucagon; (○) [25-oxindolylalanine]glucagon; (▲) [27-methionine sulfoxide]-glucagon; (⊗) [27-*S*-methylmethionine]glucagon.

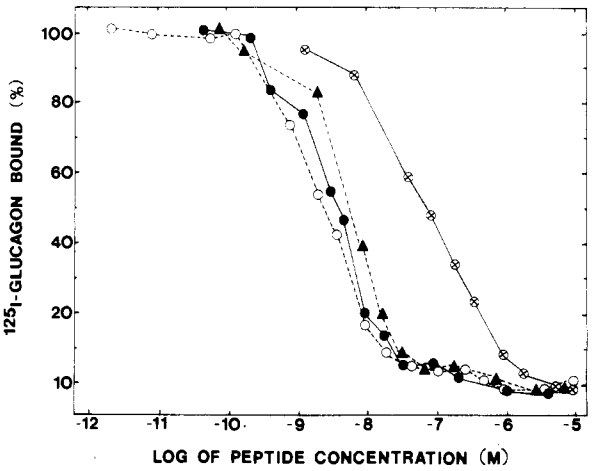

FIGURE 6: Displacement of [^{125}I]iodoglucagon bound to rat liver plasma membranes (England et al., 1982). Nonspecific binding measured in the presence of excess unlabeled peptide was 5–10% of total binding and was subtracted from the total to give specific binding. Results are expressed as the percent of maximum specific binding, which was about 1×10^5 cpm/mg of membrane protein. (●) Native glucagon; (○) [25-oxindolylalanine]glucagon; (▲) [27-methionine sulfoxide]glucagon; (⊗) [27-*S*-methylmethionine]glucagon.

glucagon was one full residue, confirming that full oxidation of the methionine had occurred.

As can be seen from Table II, one full residue of homoserine can be found in the acid hydrolysate of native glucagon cleaved by cyanogen bromide while only 0.1 nmol of homoserine was detected in 100 nmol of the peptide hydrolysate of [27-methionine sulfoxide]glucagon. From these values, the derivative was judged to be 99.9% pure. In addition, enzymatic analysis of [27-methionine sulfoxide]glucagon reveals no deamidation of asparagine or glutamine, which are believed to be important for receptor binding (Bromer et al., 1972). [27-Methionine sulfoxide]glucagon was found to be homogeneous upon isoelectric focusing and cofocused with native glucagon at a p*I* of 6.5 as would be anticipated.

Biological Characterization of the Glucagon Derivatives. The dose–response curves for the activation of rat liver adenylate cyclase by glucagon, [27-*S*-methylmethionine]glucagon, and the oxidized derivatives are shown in Figure 5. Both [25-oxindolylalanine]glucagon and [27-methionine sulfoxide]glucagon were full agonists with concentrations for half-maximal activations being 1.6×10^{-9} and 3.7×10^{-9} M, respectively. These values are close to the half-maximal concentration determined for native glucagon (2.0×10^{-9} M), suggesting that [25-oxindolylalanine]glucagon and [27-methionine sulfoxide]glucagon possess the same relative biological potency as the native hormone. These values may be compared to that of [27-*S*-methylmethionine]glucagon, whose half-maximal activation constant was 9.5×10^{-8} M, only 4.2% of that of native glucagon (England et al., 1982).

The results of the receptor binding assays are shown in Figure 6. The concentrations required for half-maximal displacement of [^{125}I]iodoglucagon were 2.3×10^{-9} M for [25-oxindolylalanine]glucagon and 4.3×10^{-9} M for [27-methionine sulfoxide]glucagon. These are in close agreement with that of native glucagon, whose concentration for half-maximal displacement was 3.2×10^{-9} M. In contrast, the concentration for half-maximal displacement by [27-*S*-methylmethionine]glucagon was 8.1×10^{-8} M (England et al., 1982), 1 full order of magnitude more than that of native glucagon or its oxidized derivatives.

Circular Dichroism. The CD spectra for native glucagon (Figure 7A), [27-*S*-methylmethionine]glucagon (Figure 7B), [27-methionine sulfoxide]glucagon (Figure 7C), and [25-oxindolylalanine]glucagon (Figure 7D) are shown in Figure 7. As illustrated, neither [25-oxindolylalanine]glucagon nor [27-methionine sulfoxide]glucagon exhibits the concentration-dependent increase in helicity at pH 10.2 that occurs in native glucagon, suggesting an inability of these derivatives to self-aggregate. These results are similar to those obtained with [27-*S*-methylmethionine]glucagon as reported earlier (Rothgeb et al., 1978) and might be anticipated since the prominent hydrophobic side chain has been replaced in each case by a more polar moiety.

Discussion

Radioiodination of polypeptide hormones is widely used for radioimmunoassays and in vitro metabolic studies. If the radiolabled hormone is to be of value for determining the ultimate fate of exogenous hormone, it must be very similar to the native molecule in biological activity and stability. Therefore, considerable interest lies in the nature of these products obtained by the iodination procedures necessary for labeling of the hormone. In addition to labeling tyrosine residues, the reagents and conditions used for the radioiodination can result in subtle alterations of several other residues, particularly the tryptophan and methionine residues. Since these residues may exert a functional role, it is important to evaluate their contribution to biological and immunological activity (Stagg et al., 1970; Shima et al., 1975).

In this paper, we have described separate oxidation procedures for tryptophan and methionine in order to ascertain what effect these alterations have upon the binding and activation capabilities of glucagon. In the preparation of [25-oxindolylalanine]glucagon, oxidation of the tryptophan causes concomitant oxidation of the methionine that can be successfully reduced to yield the single oxidation product. By chemical analysis the product has been shown to be homogeneous, to have the appropriate charge, and to contain >99% oxindolylalanine in place of tryptophan.

Following the selective oxidation of the methionine to generate [27-methionine sulfoxide]glucagon, native glucagon was removed by using a strategy of methylating the reaction mixture. Methylation of unreacted glucagon provides a chromatographic handle, allowing it to be separated from the

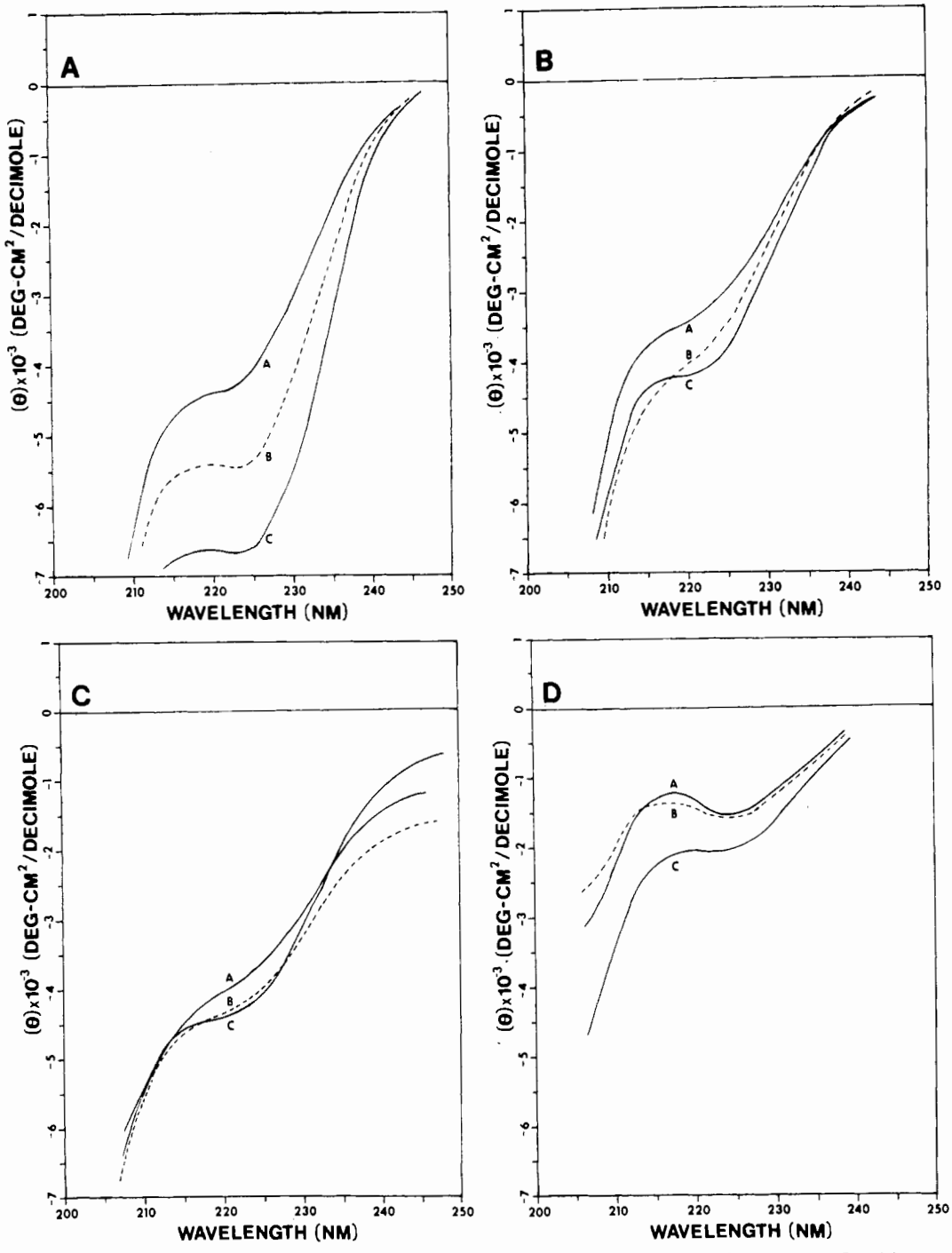

FIGURE 7: Circular dichroism spectra at pH 10.2. (A) Native glucagon: (a) 0.38 mg/mL; (b) 1.57 mg/mL; (c) 3.52 mg/mL. (B) [27-*S*-Methylmethionine]glucagon: (a) 0.39 mg/mL; (b) 1.59 mg/mL; (c) 3.05 mg/mL. (C) [27-Methionine sulfoxide]glucagon: (a) 0.43 mg/mL; (b) 1.75 mg/mL; (c) 3.50 mg/mL. (D) [25-Oxindolylalanine]glucagon: (a) 0.77 mg/mL; (b) 1.55 mg/mL; (c) 3.11 mg/mL.

desired product, [27-methionine sulfoxide]glucagon, by cation-exchange chromatography. The lack of contamination with the sulfone was shown by full reduction of the oxidized residue to methionine; quantitation of the sulfoxide was possible as a result of the protection afforded by methionine sulfoxide against cyanogen bromide cleavage (Shechter et al., 1975). This product was also shown to be homogeneous, to have the appropriate charge, and to be >99.9% pure. Assays of these derivatives indicate that both [25-oxindolylalanine]-glucagon and [27-methionine sulfoxide]glucagon are full agonists with essentially the same ability to activate adenylate cyclase and to bind to rat liver plasma membranes as native glucagon.

Circular dichroic spectra were obtained in order to examine the secondary structure for comparison with that of [27-*S*-methylmethionine]glucagon and native glucagon. Whereas native glucagon exhibits a concentration-dependent aggregation at pH 10.2 with an accompanying increase in helicity, the presence of an additional methyl group on 27-methionine and its associated positive charge has been shown to limit this aggregation (Rothgeb et al., 1978). Since [25-oxindolylalanine]glucagon and [27-methionine sulfoxide]glucagon also fail to aggregate yet are full agonists, there is a marked discrepancy between their aggregation tendencies and biological activity. We have no explanation for the reduced helicity of the derivatives at low peptide concentrations as compared with that of native glucagon. Both solubility problems and the error inherent in determining absolute values of helicity at low helix content most likely contribute to the apparent difference in secondary structure at low peptide concentrations.

Deranleau et al. (1978) have suggested that hydrophobic interactions between the 25-tryptophan and 26-leucine residues may act as a nucleation center for helix formation during aggregation. Alteration of the tryptophan side chain to the more polar oxindolylalanine moiety changes the trigonal number 3 carbon to a tetragonal configuration and so changes the relationship of the planar ring to the α and β carbons of this residue (Savige & Fontana, 1977). Such perturbations in both polarity and packing potential might be expected to decrease helix formation and intermolecular interactions. Since both [25-oxindolylalanine]glucagon and [27-methionine sulfoxide]glucagon exhibit essentially the same binding and activation as native glucagon, the results are inconsistent with the hypothesis that the hormone–receptor complex is stabilized by these interactions provided by the tryptophan and methionine side chains and may indicate that these particular residues are directed away from the receptor.

Additional studies indicate that polar interactions may play an important role in receptor binding. (Des-Asn^{28},Thr^{29})-[27-homoserine lactone]glucagon, generated by cyanogen bromide cleavage, exhibits a significant reduction in binding and biological activity relative to those of native glucagon (Lin et al., 1975). This has been interpreted in terms of a major binding contribution from the hydrophilic 28-asparagine and 29-threonine residues. Conclusions based on these results are somewhat compromised by the fact that cleavage with cyanogen bromide also converts the hydrophobic 27-methionine residue into a hydrophilic homoserine lactone residue. However, (des-Asn^{28},Thr^{29})glucagon, with its intact 27-methionine, shows no significant difference from (des-Asn^{28},Thr^{29})[27-homoserine lactone]glucagon with respect to binding affinity or biological activity (England et al., 1982), indicating that the loss of the hydrophilic forces contributes to the decreased binding affinity. Other reports are consistent with the need for conservation of polar interactions (Epand & Wheeler, 1975; Epand et al., 1981).

Other chemical modifications specifically involving the tryptophan residue result in derivatives that exhibit an equal ability to activate adenylate cyclase as native glucagon. [(2,4-Dinitrophenyl)sulfenyl][Trp]glucagon and 2-mercapto-[Trp]glucagon and its dimer were equipotent on a molar basis with native glucagon in the presence of GTP, indicating that these bulky appendages do not hinder binding of the hormone to the receptor (Wright & Rodbell, 1980). Similar results were obtained by Epand & Cote (1976). Furthermore, the photoreactive glucagon analogue 2-[(2-nitro-4-azidophenyl)-sulfenyl][Trp^{25}]glucagon of Demoliou-Mason & Epand (1982) is reported to show only a small difference in stimulating adenylate cyclase. These results also suggest that the tryptophan may not be involved in the binding process and may actually be directed away from the receptor surface. If the tryptophan side chain were directed away from the receptor, neither disruption of the hydrophobic side chain nor steric hindrance would be expected to interfere with the binding process.

Oxidation of tryptophan to form oxindolylalanine has been observed to cause significant alteration in the functional character of other proteins. In the case of lysozyme, the mode of oligosaccharide binding is altered even though 62-tryptophan does not occupy part of the binding site (Shrake & Rupley, 1980). For myoglobin, significant structural stability is lost by the oxidation, possibly as a result of the shift from a planar moiety to one with a tetrahedral configuration (Radding & Gurd, 1982). Thus, it would be anticipated that if the tryptophan residue in glucagon were involved in binding to hormone-specific receptors, a reduction in binding affinity and a corresponding loss of activity would occur upon oxidation.

Shima et al. (1975) have shown that oxidation of both the tryptophan and methionine residues in glucagon with Chloramine T results in a decrease in the immunoreactivity of the hormone toward antiserum specific for the carboxyl-terminal region. From immunoreactivity studies of cyanogen bromide cleaved glucagon, they concluded that alteration of the methionine residue is responsible for the reduced antibody binding. It is surprising then that [27-methionine sulfoxide]glucagon prepared here under similar conditions binds to hormone-specific receptors with an affinity equal to that of native glucagon. These results suggest that antibodies and receptors may recognize and bind to different faces of the hormone. This possibility could be further explored by studying the contributions of the tryptophan residue toward antibody binding with the singly oxidized derivative.

Iodoglucagon, selectively oxidized at 27-methionine and reported to be 97% homogeneous with respect to the oxidation state of the methionine side chain, has been shown to differ in its affinity toward both isolated adipocytes and hepatocytes as compared with the nonoxidized iodoglucagon (Sonne et al., 1982). These results are in contrast to the binding studies reported here in which [27-methionine sulfoxide]glucagon binds similarly to native glucagon. However, since the values for the average binding affinities were reported only as ratios of the nonoxidized/oxidized forms of iodoglucagon, a direct comparison of the binding affinity of [27-methionine sulfoxide]iodoglucagon with that of the [27-methionine sulfoxide]glucagon reported here is not possible.

In other studies of the bioactivity of [27-methionine sulfoxide]glucagon, a decreased potency for glucose mobilization in hepatocytes has been reported (Nooijen & Kempen, 1979). This finding and our results suggest the possibility that two receptor populations exist. The receptor coupled with adenylate cyclase activity appears to be insensitive to methionine oxidation whereas a second receptor mechanism responsible for glucose mobilization in intact cells is sensitive to methionine oxidation.

Oxidation of certain amino acids by radioiodination has been suggested to be responsible for the covalent cross-linking of such peptides as epidermal growth factor and insulin to their receptors (Saviolakis et al., 1981; Comens et al., 1982). The selective oxidation strategies described in this paper could be used to clarify which oxidizable side chains are involved in the cross-linking phenomenon. Oxidative damage incurred during radioiodination has also been linked to diminished or abolished biological activity in other peptide hormones such as gastrin and bovine parathyroid hormone (Stagg et al., 1970; Keutmann et al., 1971). However, our findings that the singly oxidized derivatives, [25-oxindolylalanine]glucagon and [27-methionine sulfoxide]glucagon, have adenylate cyclase activities quantitatively similar to that of native glucagon suggest that mild oxidative damage to these residues during the radioiodination of glucagon is less critical than might have been anticipated. Although excessive exposure to Chloramine T may cause multiple oxidations within the molecule leading to an altered biological potency, these mild oxidation reactions are among the few procedures for the modification of amino acid side chains in glucagon that do not alter the binding and activation potential of the hormone.

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